Amyloid fibril formation and protein misassembly: a structural quest for insights into amyloid and prion diseases

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The assembly and misassembly of normally soluble proteins into fibrilar structures is thought to be a causative agent in a variety of human amyloid and prion diseases. Structural and mechanistic studies of this process are beginning to elucidate the conformational changes required for the conversion of a normally soluble and functional protein into a defined quaternary structure.

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Structure 15 May 1997, 5:595–600
http://biomednet.com/elecref/0969212600500595

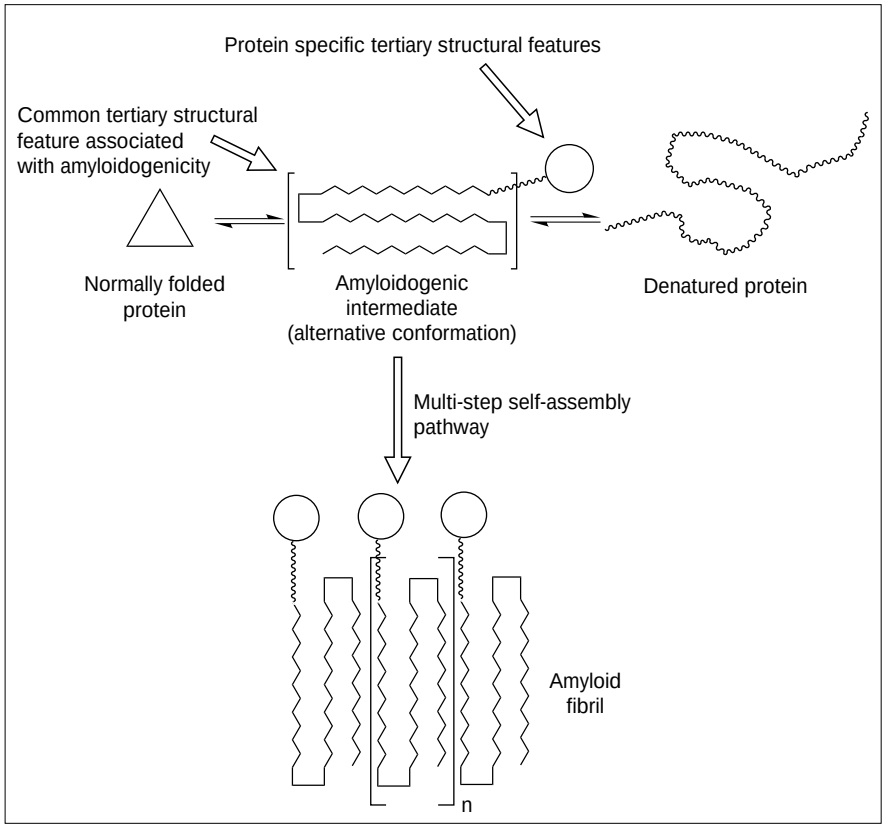
Protein assembly and misassembly processes are difficult to characterize by conventional X-ray crystallography and multidimensional solution NMR studies owing to the non-crystallinity and the insolubility of the fibrilar form of the assembled protein [1,2]. However, these methods have a very important role to play in characterizing both the structure of the native fold of the precursor protein and the tertiary structure(s) of the intermediate(s) that render a given protein capable of self-assembly into an amyloid fibril. Moreover, by creatively modifying the precursor protein it is possible to arrest the assembly process at an intermediate stage such that an X-ray structure of an assembly intermediate can be determined [3–5]. The fibrilar structure resulting from the assembly of an alternative conformation of any one of the 16 normally soluble and functional human amyloidogenic proteins is thought to be the causative agent in a variety of human amyloid and prion diseases [6–17]. Support for the prion hypothesis comes from research on yeast where the self-assembly of the N-terminal domain of Sup35 is responsible for the dominant cytoplasmically inherited [PSI$^+$] phenotypic trait [18]. [PSI$^+$] cells have the ability to read through the nonsense codons present in certain genes, resulting in the synthesis of novel proteins [19,20]. As Sup35 is a subunit of the translation release factor that allows ribosomes to terminate translation at nonsense codons, it has been argued that the [PSI$^+$] phenotype is simply another mechanism for yeast to adapt to stressful environments by producing a new subset of proteins. It will be very interesting to see how many examples there are of intracellular proteins which self-assemble and provide genetic variation to a cell.

There are two basic types of protein self-assembly mechanisms. Assembly can occur from a conformational intermediate produced during folding or denaturation, as is the case for inclusion body formation and amyloid fibril formation (Fig. 1), respectively [6,21,22]. Alternatively, the native three-dimensional structure of a protein can become susceptible to self-assembly through surface mutations, as in the case of Sickle cell hemoglobin, or due to a change in environment analogous to the conditions facilitating protein crystallization [23]. X-ray crystallography is easily applied in understanding the latter case, where the normal tertiary structure facilitates self-assembly through quaternary contacts, but does not apply directly to characterizing the intermediate structure(s) in the former case. In this minireview we use the terms assembly and misassembly to characterize ordered quaternary structure formation and reserve the term aggregation for those cases where a protein forms a non-regular amorphous oligomeric structure.

Amyloid fibril formation refers to an *in vivo* self-assembly process where any one of 16 normally soluble and functional human proteins are transformed into an insoluble, cross-β fibrilar quaternary structure. The fibrils derived from different precursor proteins have similar structural features, despite only modest primary and tertiary structural homology amongst the precursor proteins [1,6,7,24,25]. The low structural homology is reflected in the apparently unrelated functions of the amyloidogenic proteins *in vivo*. For example, transthyretin (TTR) transports thyroxine and the retinol-binding protein in the cerebral spinal fluid and in the blood, whereas lysozyme is an enzyme that destroys bacterial cell walls. Biophysical studies on TTR [26–32] and variants thereof [33,34], immunoglobulin light chains [12], and more recently lysozyme [11], highlight the requirement of conformational changes for the conversion of these proteins into amyloid fibrils [6,8]. Under partially denaturing conditions, amyloidogenic proteins appear to be able to adopt alternative conformations which render these proteins capable of self-assembly into amyloid fibrils [6]. The observation that apparently unrelated proteins can form amyloid having a common tertiary and quaternary structure can be rationalized by considering the possibility that a portion of the structure formed under partially denaturing conditions is a common core β sheet that allows this subset of proteins to assemble into the amyloid quaternary structure. The amorphous regions of amyloid derived from different proteins are likely to vary, however, not much information is available on the amorphous regions at this time. The mechanism governing the assembly of the amyloidogenic intermediate into amyloid is rather complex

Figure 1

Outline of the role that conformational changes play in the conversion of a normally soluble and functional protein into amyloid. Amyloidogenic proteins appear to adopt alternative conformations which have common structural features.



and involves several discernible quaternary structural intermediates (see references [6,35–40]).

To outline what can be achieved with respect to a structural understanding of protein assembly, the next section focuses on our current understanding of TTR amyloid fibril formation. Our present knowledge of this process was obtained employing a combination of biophysical and structural methods. It can be argued that TTR and its associated amyloid diseases are better understood than any other human amyloid disease [6,7,27,32,41–48]. In unfortunate individuals wild-type TTR is converted into amyloid which appears to cause senile systemic amyloidosis (SSA). The age of onset is about 80 and the disease is sometimes characterized by heavy amyloid deposits in the heart leading to congestive heart failure [49,50]. Familial amyloid polyneuropathy (FAP) occurs much earlier in life than the senile form of the disease, onset being as early as the second decade of life in the case of one variant (Leu55→Pro) [45,51–53]. FAP patients are predominantly heterozygous individuals having one of over 50 single site variants of TTR in their systemic amyloid deposits. All of the early onset TTR amyloid disease cases observed to date are associated with a mutation in TTR, implying that the mutation makes the protein more amyloidogenic. The majority of the FAP mutations are conservative surface mutations and as such would not be expected to change the overall three-dimensional structure of TTR [54].

The structure of wild-type TTR was determined by Blake and his colleagues at Oxford in the 1970s [55,56]. One possibility to explain the amyloidogenicity of the variant FAP forms of TTR, relative to the wild-type, is that the mutations simply allow the normally folded TTR tetramers to undergo self-assembly, analogous to the sickle cell hemoglobin situation. However, several experiments demonstrate that TTR tetramers do not form amyloid, even when the tetrameric FAP variants are incubated well above physiological concentrations. The X-ray crystal structures of several of the FAP-associated TTR variants have now been solved, including the most common Val30→Met mutant. These variants exhibit structures which are virtually identical to the structure of the wild-type protein [57–60]. This is not surprising, given the conservative nature of the majority of these mutations [54].

If the mutations do not affect the normally folded structure then they are likely to perturb either the thermodynamics and/or the kinetics of the folding/denaturation pathway [6,8,10,11,61,62]. Wild-type TTR will form

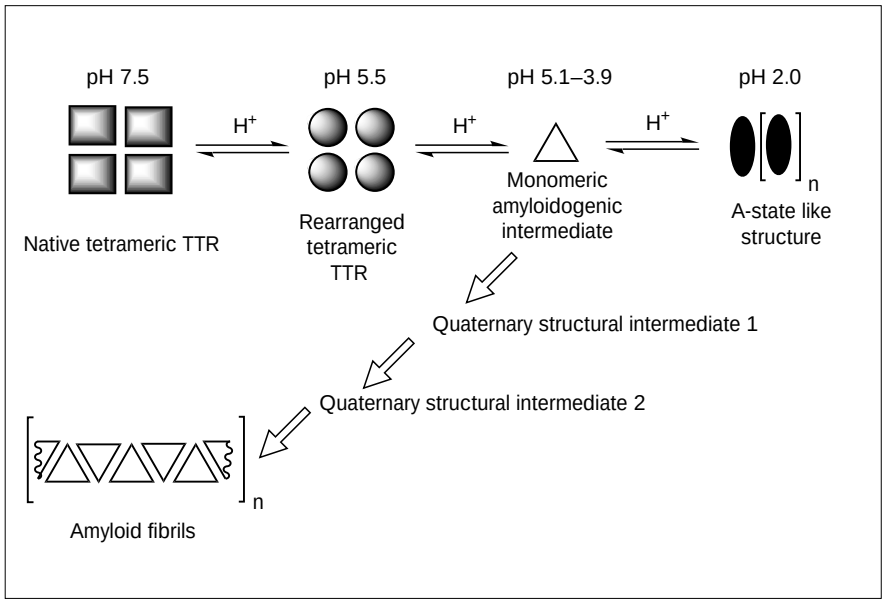
amyloid *in vitro* when subjected to partial acid denaturation conditions (within the pH range 5.1–3.9) and during pH-mediated reconstitution from an unfolded state (pH 3.9–5.8). These observation suggest that an intermediate of the denaturation/reconstitution pathway partitions into amyloid at physiological TTR concentrations (0.2–0.3 mg ml^{-1}) and temperature (37°C) [26,27,29–34]. The FAP TTR variants make more amyloid than the wild type and exhibit fibril formation within a higher pH range, strongly suggesting that the mutations do indeed alter the denaturation pathway [27,29,33,34]. The fact that these mutations allow amyloid to form over a higher pH range is physiologically important, because protein turnover normally takes place at pH 5.5 [6,33,34,63]. If fibril formation is efficient under normal protein turnover conditions, affected individuals should develop amyloid disease much earlier in life due to the large mass of amyloid accumulated. The tertiary and quaternary structure of the TTR intermediate(s) that lead to amyloid fibril formation have been identified by unlinking denaturation and fibril formation [29]. Analytical ultracentrifugation studies are the principal method used for evaluating quaternary structural changes as a function of pH, whereas spectroscopic methods (such as near and far UV circular dichroism and fluorescence spectroscopy in combination with single tryptophan containing variants) facilitate characterization of the pH-dependent tertiary structural changes [27,29–31]. These methods lead us to the denaturation pathway shown in Figure 2. The data accumulated suggest that TTR must dissociate into monomeric form and undergo a tertiary structural change in order to be competent to self-assemble into amyloid [6].

Only very recently have conditions been identified which allow TTR to be stabilized in its monomeric amyloidogenic intermediate conformation for possible multidimensional NMR evaluation. Dobson, Wuthrich, Dyson, Wright, Baum, and others have clearly demonstrated that, while difficult, NMR structure determination on intermediates of this type is feasible and provides a wealth of information [64–66]. By N-methylating critical hydrogen-bonding residues or through equivalent modifications, it is possible to block self-assembly at an early oligomer stage, preventing the formation of fibrils and making the structure amenable to crystallographic structure determination [3].

From emerging studies employing atomic force microscopy, electron microscopy, and analytical ultracentrifugation methods it is now apparent that fibril quaternary structures are quite complex [35–39]. These techniques have been used to observe multiple quaternary structural intermediates prior to the formation of amyloid fibrils. Because quaternary structural interactions are very important in fibril formation, caution must be exercised when evaluating an assembly terminated amyloid structure, such as a dimeric structure. Such a structure may lack the quaternary interactions that are characteristic of the true amyloid structure. These results also force one to consider the possibility that one or more of these soluble quaternary structural intermediates may play a key role in the etiology of amyloid disease [6,32]. Undoubtedly, methods will be discovered (for example those utilizing small molecule inhibitors) that block the conversion of one quaternary structure into the next, allowing us to unlink the assembly equilibria characteristic of amyloid formation.

Figure 2

The pH-dependent denaturation/amyloid fibril formation pathway exhibited by transthyretin (TTR).



The key to the structural characterization of amyloid fibrils by X-ray diffraction analysis is predicated on orienting the fibrils [2]. Blake and his colleagues at Oxford were able to isolate oriented Val30→Met TTR fibrils from the vitreous humour of a homozygous FAP patient. These fibrils were studied employing a synchrotron radiation source using partially dried samples mounted on a stretch frame. A high angle diffraction pattern was observed showing meridional reflections out to 2 Å resolution [2]. This pattern is consistent with a novel cross-β helical structure, having 24 β strands per turn of the β helix which extends indefinitely in the helical axis direction. It appears that the β-helical protofilament is four twisted β-helices thick (50–60 Å in diameter), although the orientation of the individual β strands cannot be discerned from the data. Four β-helical protofilaments further associate to form a fibril, as discerned from the cross-sectional views observed in electron microscopy studies (130 Å in diameter) [2,67]. Consistent with the X-ray diffraction data, the proposed structural model of amyloid requires a considerable tertiary structural rearrangement; this is in agreement with the rearrangement suggested by independent biophysical data and V8 proteolysis sensitivity data [6]. Although the proposed β-helical structure of TTR amyloid is consistent with a number of observations, it is based on a small number of wide-angle reflections, as pointed out by Kirschner. As such, further experiments are needed to provide more experimental evidence for the β-helical structural model of amyloid [68].

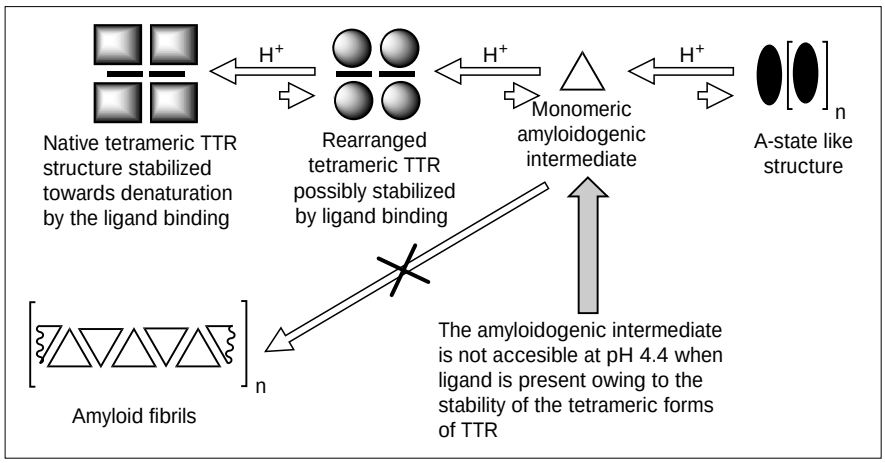
In summary, structural information on the precursor proteins, the structurally altered monomeric intermediates, the quaternary structural assembly intermediates, and the amyloid fibrils themselves will be very useful for improving our understanding of protein assembly and misassembly in general and specifically in the case of amyloid fibril formation.

Recent examples of the application of structural methods to study protein assembly include Wuthrich's structure of the PrP 121–231 domain of the prion precursor protein. This structure contains the residues that are changed in nine of the familial prion diseases as well as two glycosylation sites and the disulfide bond [69]. The PrP domain structure should represent the normal structure of the prion protein, however, one needs to realize that neither glycosylation site is post-translationally modified, which could change the structure of the precursor protein. The crystal structures of the native folds of both of the amyloidogenic variants of lysozyme have recently been reported. These structures show changes in the critical interface between the α and β domains, as compared to the wild-type protein, which could enhance their amyloidogenicity. In addition, the UK group headed by Pepys has used a number of biophysical methods to show that an intermediate molten globule state is the lysozyme intermediate that leads to amyloid fibril formation [11]. Studies on the time course of Alzheimer's fibril formation by the Krafft and Lansbury groups, have shown that before the mature fibrils appear *in vitro* at least two quaternary structural intermediates accumulate during Alzheimer's β-amyloid fibril formation [39,40]. Studies such as these will continue to increase our understanding of protein self-assembly. The Lansbury and Griffin laboratories as well as the Kirschner laboratory have proposed structural models for the Alzheimer's β-amyloid using solid state NMR and X-ray diffraction methods, respectively [70,71]. Continuing efforts from these and other laboratories should result in a high-resolution model for the Alzheimer's amyloid fibril.

The role of structural methods in developing therapeutic strategies for intervention in amyloid disease is particularly attractive [72]. The mechanism of TTR denaturation, depicted in Figure 2, suggests that amyloid fibril formation could be inhibited in two ways: by preventing

Figure 3

Ligand binding stabilizes the normal fold of the amyloidogenic protein transthyretin (TTR), thus preventing the formation of the alternative conformation which leads to amyloid fibril formation.



the conformational changes leading to the formation of the amyloidogenic intermediate or by interfering with the process of assembly [6,72] (Fig. 3). Preventing the conformational changes has proven to be straightforward using molecules like 2,4,6-triiodophenol which bind with high affinity to TTR [72,73]. Ligand binding stabilizes the tetramer against the conformational changes that lead to amyloid fibril formation at pH 4–5.5 [72]. A recent structure-based design approach has led to the production of a number of very interesting compounds which inhibit TTR fibril formation *in vitro*. Two of these compounds have the appropriate pharmacodynamics to warrant evaluation in FAP patients (SA Peterson, T Klabunde, H Lashuel, JC Sacchettini and JWK, unpublished data). These new approaches towards the treatment of conditions such as FAP hold much promise for the treatment of amyloid diseases in the future.

Acknowledgements

We wish to thank the National Institutes of Health (R29 DK46335-01) as well as the Alzheimer's Association, Vivian L Smith Foundation Pilot Research Grant, and the Regina M Ackermann Memorial Fund for financial support of the Kelly Laboratory research discussed in this minireview.

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